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Zinc-nitrogen co-fertilization influences N_2O emissions and microbial communities in an irrigated maize field

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ABSTRACT

Previous studies have shown that the use of zinc (Zn) chelate fertilizers combined with a nitrogen (N) fertilizer (urea) can lead to both agronomic (i.e., yields and Zn and N biofortification due to the synergies between both nutrients) and environmental (i.e., by reducing the emissions of nitrous oxide, N_2O, derived from N fertilization) benefits under rainfed semi-arid conditions. However, little is known about the effect of Zn-N co-fertilization on greenhouse gas (GHG) emissions or soil microbial processes involved in N_2O fluxes under non-flooded irrigated conditions (during the dry season). Under these conditions, water-filled pore space continuously fluctuates following a periodic pattern and soil temperatures are in the optimum range for soil microorganisms. In this context, a field experiment was conducted using a maize (*Zea mays* L.) crop treated with two N levels (no N application and 120 kg N ha^{-1} as urea), and three Zn sources (no Zn application, Zn sulphate, and Zn applied with a mixture of chelating compounds, DTPA-HEDTA-EDTA). Nitrous oxide, methane (CH_4) and carbon dioxide (CO_2) fluxes were measured using opaque chambers, as well as the total abundances of soil bacteria, archaea and nitrifier and denitrifier communities. Zn-N co-fertilization increased cumulative N_2O emissions from 0.36 kg N-N_2O ha^{-1} (for urea combined with Zn chelates) to 0.76 kg N-N_2O ha^{-1} (for urea combined with Zn sulphate), with respect to urea without Zn application. The N_2O emission factors were lower (0.34%–0.72%) than the IPCC default value of 1%. Total abundances of the *nosZ* denitrification gene, which is involved in the reduction in N_2O to dinitrogen (N_2), were reduced by 75% on average in the plots that received Zn fertilizers. This reduction may explain the higher N_2O emissions in these treatments. In contrast with the case with non-irrigated crops, Zn-N co-fertilization cannot be recommended as a strategy to mitigate N_2O emissions in irrigated maize under semi-arid conditions, despite of the enhancement of Zn availability in soil.

1. Introduction

Sustainable agriculture aims to increase food production in the context of a growing worldwide population without compromising crop quality or yield and while reducing environmental pollution (Billen et al., 2015; Quemada et al., 2020; Spiertz, 2010). Inputs of N, which are necessary to maintain crop yield, contribute to the release of nitrous oxide (N_2O), a potent greenhouse gas (GHG) (Venterea et al., 2012). In turn, this contribution is speculated to increase the global warming potential associated with agricultural systems (Robertson et al., 2000). To mitigate this negative effect, several fertilization strategies have been proposed based on the "4R" approach (which means applying N fertilizers with the right rate, right placement, right time and right source) (Li et al., 2019). In addition to improved N fertilization, strategies based on the management of water and balanced fertilization with macro- and micronutrients should also be considered due to the synergistic effects on nutrient uptake (Fageria and Baligar, 2005).

Zinc (Zn) fertilization is recommended for cereal crops, especially in Zn-deficient soils, to increase Zn concentration in grain (biofortification). Zinc biofortification alleviates the deficiency of this element in the human diet, which is very frequent in widespread areas of the world, especially when local diets are based on cereals (Hotz and Brown, 2004; Stein, 2010). Zinc has been recognized as one of the main target micronutrients, since its supplementation is associated with

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reductions of the incidence of infectious diseases such as pneumonia, particularly among children in areas with insufficient Zn supply (Gibson, 2012). A synergistic effect of Zn in the soil N cycle has been discovered, since this metal serves as a cofactor for some enzymes involved in N metabolism (Glass and Orphan, 2012). Moreover, the enhancement of biomass yield as a result of Zn application could also have a positive effect on N acquisition (Montoya et al., 2020), thus reducing potential N losses. Almendros et al. (2019) also observed this synergistic effect in the yield of a rainfed barley crop, applying Zn with a mixture of synthetic chelating compounds and urea as the N source. In addition, Asif et al. (2013) and El-Badawy and Mehasen (2011) reported that fertilization with Zn sulphate combined with N in the form of urea or ammonium nitrate, respectively, can positively affect the yield and the yield components in a maize crop.

Within the N cycle, nitrification and denitrification are considered to be the two main biological processes leading to the release of N_2O from soils (Butterbach-Bahl et al., 2013). Nitrification mainly occurs under aerobic conditions, whereas denitrification predominates under oxygen-limited conditions, albeit the two processes often occur simultaneously rather than in an isolated way when the nearby co-existence of both oxic conditions and anaerobic microsites occurs (Baggs and Philippot, 2011; Hallin et al., 2018). During nitrification, ammonium (NH_4^+) is oxidized to nitrite (NO_2^-) by the enzyme ammonia monooxygenase, and N_2O is produced as a secondary product; NO_2^- is further converted to nitrate (NO_3^-) by the nitrite oxidoreductase enzyme. The ammonia-oxidizing archaea (AOA) and bacteria (AOB) contain the *amoA* gene encoding ammonia monooxygenase, which has been reported to require copper (Cu) and quite possibly Zn and iron (Fe) for its activation (Ensign et al., 1993; Glass and Orphan, 2012). Denitrification involves the reduction of NO_3^- to dinitrogen (N_2) through the stepwise formation of NO_2^-, NO and N_2O. These reactions are catalysed by the enzymes nitrate (NapA/NarG)-, nitrite (NirK/Nirs)-, nitric oxide (Nor)- and nitrous oxide (Nos)-reductases encoded by the *napA/narG*, *nirK/nirS*, *norB* and *nosZ* genes, respectively (Bueno et al., 2012; Butterbach-Bahl et al., 2013; Hallin et al., 2018). NarG contains a [4Fe-4S] cluster, NirS is a homodimeric protein with hemes *c* and d_1, and NirK and NosZ are Cu-containing enzymes (Bueno et al., 2012 and references therein).

To date, few studies have assessed the influence of Zn availability and Zn fertilizers on the biochemical reactions involved in N_2O production and consumption in soils, and a clear and consistent effect of the addition of this element has not been found so far. For instance, the addition of Zn to a mangrove soil inhibited nitrification and decreased N_2O losses (Chen et al., 2014). In rainfed wheat, Montoya et al. (2018) showed that the effect of Zn was very dependent on the Zn source and synthetic Zn-chelating compounds (i.e., a mixture of diethylenetriaminepentaacetate (DTPA), hydroxyethyl-ethylenediaminetriacetate acid (HEDTA), and ethylenediaminetetracetate acid (EDTA)) decreased the total abundance of *nirK*, *nirS*, and *amoA* genes but increased that of the *nosZ* gene. This effect produced a short living but significant mitigation of N_2O emission in comparison with the other Zn sources (including Zn sulphate, $ZnSO_4$) and no Zn application. In contrast, application of a synthetic chelating agent (EDTA) to a submerged paddy crop produced a significant increase in N_2O emissions (Pramanik and Kim, 2017) due to an enhancement of the denitrification process.

To our knowledge, there are no studies evaluating the effects of Zn fertilizers or Zn-N co-fertilization on N_2O emissions and related microbial populations in non-flooded irrigated crops, where frequent temporal variations in moisture content occur concurrently with high soil temperatures, affecting both nitrification and denitrification processes. In addition, little is known about the effect of Zn-N co-fertilization on crop yields or Zn biofortification in irrigated maize systems. The objectives of this field experiment were, therefore: (i) to establish the links between the sources of Zn, N_2O emissions and nitrification and denitrification gene abundances, and ii) to assess the potential synergetic effect of Zn and N fertilizers on Zn biofortification. Our initial hypothesis was that Zn-chelating compounds would mitigate total N_2O emissions (Montoya et al., 2018), because of reduction in the abundances of the N_2O producers (AOA, AOB, and those containing the *nirK* and *norB* genes) and an increase in numbers of N_2O reducers (*nosZ* gene), also leading to the enhancement of Zn concentrations in grain.

Table 1
Selected soil properties measured in this study.

Soil Properties	Data
Bulk density (g cm^{-3})	1.27
Clay (%)	10
silt (%)	59.5
sand (%)	30.5
pH_{water} (1:2.5, w/v)	8.2 ± 0.03
Oxidizable OM (g kg^{-1}) (Walkley-Black procedure)	20.7 ± 0.45
Extractable P (mg kg^{-1}) (Olsen extraction procedure)	28.4 ± 0.62
Total N (g kg^{-1})	1.64 ± 0.12
N-NO_3^- (mg kg^{-1})	27.4 ± 3.29
DTPA-TEA-Extractable Metal (mg kg^{-1}):	
Zn	0.85 ± 0.14
Cu	0.79 ± 0.13
Fe	5.62 ± 1.14

Means of three replicates ± standard deviation. (*DTPA-TEA*, diethylenetriaminepentaacetate-triethanolamine).

2. Material and methods

2.1. Field site and soil characterization

The experiment was conducted at "Centro Nacional de Tecnología del Regadío" (Madrid, Spain) in a *Typic xerofluvent soil* (Soil Survey Staff, 2017). The general properties of the topsoil are reported in Table 1. This soil is slightly deficient in Zn, with DTPA-Zn < 1 mg kg^{-1} (Brennan et al., 1993). The site has a Mediterranean climate and annual rainfall and air temperature (10-year average data) were 384 mm and 14.2 °C, respectively. Maximum temperatures reached in the months of July and August of the last 10 years (on average) were 37 °C and 38 °C, respectively. Data for daily rainfall and soil temperatures (at 10 cm soil depth) from a meteorological station located at the farm were downloaded from http://eportal.mapama.gob.es/websiar/Inicio.aspx.

2.2. Experimental design and management

The study was conducted during one maize (*Zea mays* L.) cropping season. The experimental field was sown at a density of 9.5 plants m^{-2} on 18 April 2017. A total of 18 plots (12 m × 12 m) were arranged in a triply replicated split plot design with two N rates (i.e., control without N application, N0; and urea, U) as the levels of the main factor and three Zn sources as levels of the second factor (subplots) (i.e., control without Zn application, Zn0; Zn sulphate 35% Zn w/w, ZnSul; and Zn applied with a mixture of chelating compounds DTPA-HEDTA-EDTA 7% Zn w/w, ZnCh). Both main plots and subplots were completely randomized within the split plot design.

Nitrogen fertilization was carried out in V6 stage (Ritchie et al., 1982) by applying 200 kg N ha^{-1} as urea (provided by EuroChem Agro) on the soil surface. Foliar Zn fertilizers were sprayed by hand on two different occasions using a knapsack sprayer (foliar-soil application), half of the total amount (5 kg Zn ha^{-1} for ZnSul, 0.15 kg Zn ha^{-1} for ZnCh) at the V6 stage and the other half at the VT stage. Consequently, the total quantity of Zn applied was 10 kg Zn ha^{-1} for ZnSul (a similar rate to that used by Gonzalez et al. (2019) under Mediterranean conditions) and 0.30 kg Zn ha^{-1} for ZnCh, following the recommendations of the manufacturers (De Liñán-Carral and De Liñán-Vicente, 2016). During foliar application, a portion of the Zn compound falls to soil (foliar-soil application). An irrigation event was implemented the day

after each fertilizer application. The maize plants were harvested on 20 September. Preemergence herbicide (a mixture of Spectrum®, 3 l ha^{-1}; and Stomp® Aqua, 1.25 l ha^{-1}; provided by BASF) treatment was only applied at seeding.

Maize crop was irrigated with a total amount of 660 mm in 36 irrigation events through a ranger irrigation system (length: 160.5 m, distances between sprinklers: 4 m and total flow: 68400 l h^{-1}). The calculation of the water dose was performed as described by (Allen et al., 1998).

2.3. Greenhouse gas flux measurements

Gas samples were collected 2–3 times per week during the first month after fertilization events; in addition, a sample was taken prior to fertilization. The frequency of sampling was diminished progressively, whilst ensuring that all soil rewetting events were covered and following the suggestions (regarding sampling time and frequency) of Reeves and Wang (2015). Fluxes of the greenhouse gases N_2O, CH_4 and CO_2 were measured using opaque manual circular static chambers (Abalos et al., 2012) following the methodology described by Davidson et al. (2002). Gas samples were taken at 0, 30 and 60 min to test the linearity of gas accumulation in each chamber. The increases in the GHG concentrations within the chamber headspace were generally linear (> 90% of cases) during the sampling period. In the case of nonlinear fluxes, linear regressions were performed, since this has been described as the recommended procedure (relative to non-linear regression) by Lammirato et al. (2018) and Venterea et al. (2012). Concentrations of N_2O, CO_2 and CH_4 were determined with a gas chromatograph equipped with two detectors (ECD and FID) as described in Recio et al. (2018).

2.4. Soil and plant sampling and analyses

Soil samples were collected at 0–10 cm depth with the same sampling frequency as that for GHGs. Soil moisture, but not mineral N, was analyzed in all soil sampling events. Cylindrical cores (2.5 cm diameter and 12 cm length) were used to take the soil samples, and three samples per plot were randomly collected to obtain a representative mix. Soil NH_4^+ and NO_3^- concentrations were measured from soil extracts (8 g of soil in 50 mL of 1 M KCl) by UV-V spectrophotometry, using the equipment described by Guardia et al. (2018). The soil water-filled pore space (WFPS) was estimated as explained by Abalos et al. (2012), after measuring the gravimetric water content by oven-drying the soil samples.

The soil concentrations of Zn and Cu available for plants were determined using the DTPA-TEA method (5 mM DTPA + 10 mM $CaCl_2$ + 0.1 M TEA adjusted to pH 7) on four different occasions: before fertilization, after each fertilization event and at harvest (Lindsay and Norvell, 1978). The extraction of micronutrients was accomplished with 20 mL of the DTPA-TEA solution and shaking for 2 h. Each suspension was filtered through Filter-Lab number 1246 quantitative filter paper, and the filtrate was measured by flame atomic absorption spectroscopy (FAAS) (Perkin-Elmer AAnalyst 700).

At maize harvest, two central rows (a total of 10 m) in each plot were collected and weighed in the field. The plants of each row were air-dried, and two different fractions of plants were obtained, grain and stover. Then, these fractions were ground and sieved at 2 mm to determine the total N (by TruMac CN Leco elemental analyser) and Zn contents in each one. Total extraction of Zn from dry plant matter was performed by wet acidic digestion (10 mL of HNO_3 (65%), 10 mL HCL (37%) and 10 mL of deionized water) overnight, and then the sample was kept in a digester (SPB 50–24 with an SPB digital, Perkin-Elmer) for 2 h at 140 °C. Finally, the sample was filtered through Filter-Lab number 1244 quantitative filter paper into a 50-mL graduated flask, and the determinations were performed by FAAS.

2.5. Soil DNA extraction and abundances of nitrifier and denitrifier communities

Soil samples for DNA determinations were taken on three different dates related to the N_2O fluxes [before peak (06/19/2017), after peak (07/04/2017) and at the end of the field experiment (07/17/2017)]. DNA extraction was performed in 0.5 g of soil using the commercial PowerSoil® DNA isolation kit (Qiagen); the concentration was determined using the Qubit® ssDNA assay kit (Molecular Probes) and stored at −20 °C until use.

Quantitative PCR (qPCR) was used to estimate the size of the nitrifier community after amplification of the *amoA* gene from ammonia-oxidizing bacteria (AOB) and archaea (AOA) and size of the denitrifier community by qPCR of the *nirK*, *norB* and *nosZ* genes using primers and thermal conditions previously reported (Montoya et al., 2018). The corresponding 16S rRNA gene was used as a molecular marker to quantify the total bacterial (16SB) and archaeal (16SA) communities. The qPCR assays were achieved using an iQ5 Thermocycler (Bio-Rad Laboratories, USA) with SYBR Green as the detection system. PCR efficiency for the different assays ranged between 90% and 99%. The quality of all qPCR amplifications was verified by electrophoresis in agarose and by melting curve analysis.

2.6. Calculations and statistical analysis

Linear interpolations between sampling dates were carried out to calculate the cumulative gas emissions. The CO_2 equivalent (CO_2eq) emissions of the N_2O and CH_4 fluxes was calculated using the climate-feedback corrected values from Pachauri et al. (2014) over a 100-year time horizon; i.e., 298 for N_2O and 34 for CH_4. The GHG intensity (GHGI) was calculated as the ratio of CO_2eq emissions to grain yield.

Analyses of variance (two-way ANOVAs) were performed with *Statgraphics Centurion XVII* for abiotic (soil NH_4^+ and NO_3^- concentrations, N_2O, CH_4 and CO_2 cumulative fluxes, CO_2eq emissions, total Zn and N contents in grain and stover, total Zn and Cu-DTPA in soil) and biotic (total abundances of genes) factors. Normality of distributions and variance uniformity were tested beforehand as explained by Guardia et al. (2018), and $\log_{10}$ transformation and a non-parametric test (Kruskal-Wallis) were used for average soil NH_4^+ and NO_3^- concentrations, respectively. Significant differences between treatments were identified using the Least Significant Difference (LSD) test at $P < 0.05$.

3. Results

3.1. Environmental conditions and mineral N and trace metals in soil

The average daily mean soil temperature during the maize-cropping cycle was 20.9 °C (22.9 °C the first month after N fertilization and 23.6 °C during the reproductive period) (Fig. 1). The maximum temperature exceeded 35 °C for 40 days from mid-June to the end of August, of which 16 days corresponded to the crop reproductive period. In addition, the maximum temperatures reached in July and August were 40 °C and 39 °C, respectively, these being values higher than averages of the values from the last 10 years (see Section 2.1). The WFPS ranged from 15% to 86%, showing continuous fluctuations due to irrigation events and drying episodes (Fig. 1b).

Soil NH_4^+ and NO_3^- concentrations for each treatment are shown in Fig. 2. The N-fertilized treatments exhibited significantly greater NH_4^+ content than did treatments without N fertilizer. The NH_4^+ concentration decreased rapidly 5 days after fertilization (DAF, Fig. 2a), and subsequently the NO_3^- content was elevated (Fig. 2b). The Zn sources did not cause any effect on the mineral N content in the topsoil.

Total Zn-DTPA concentrations were 60% and 13% higher (on average) in the soils treated with ZnSul and ZnCh, respectively, than in the Zn0 treatment on both sampling dates (Table 2). The Zn availability

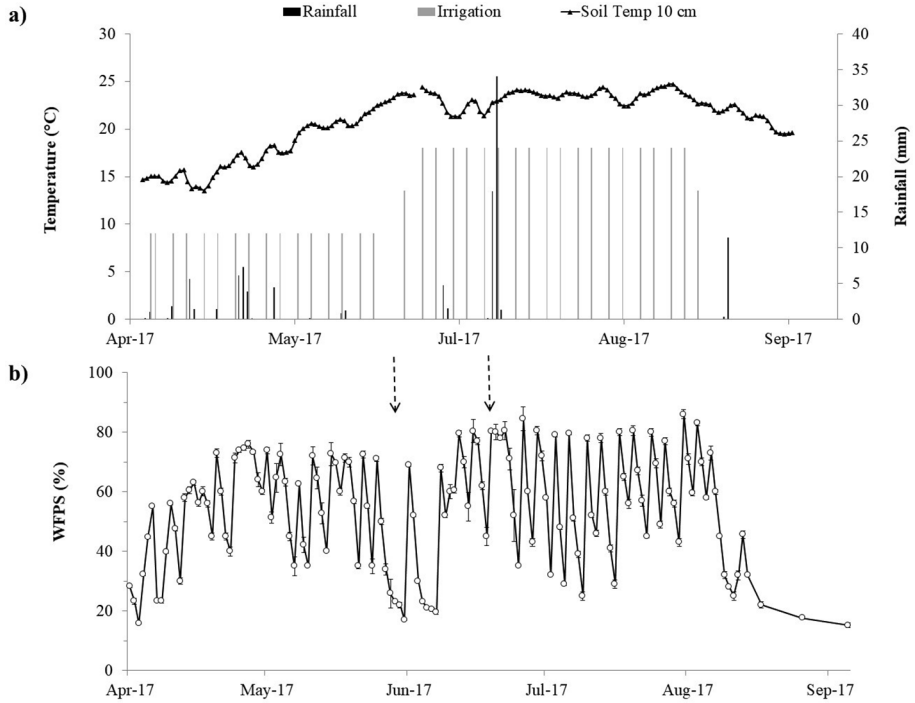

Fig. 1. a) Daily rainfall (mm) and mean soil temperature at 10 cm during the experimental period. b) Soil moisture content expressed as water filled pore space (WFPS %) during the experimental period. The dotted arrows indicate the Zn fertilizations events. Vertical bars indicate standard errors.

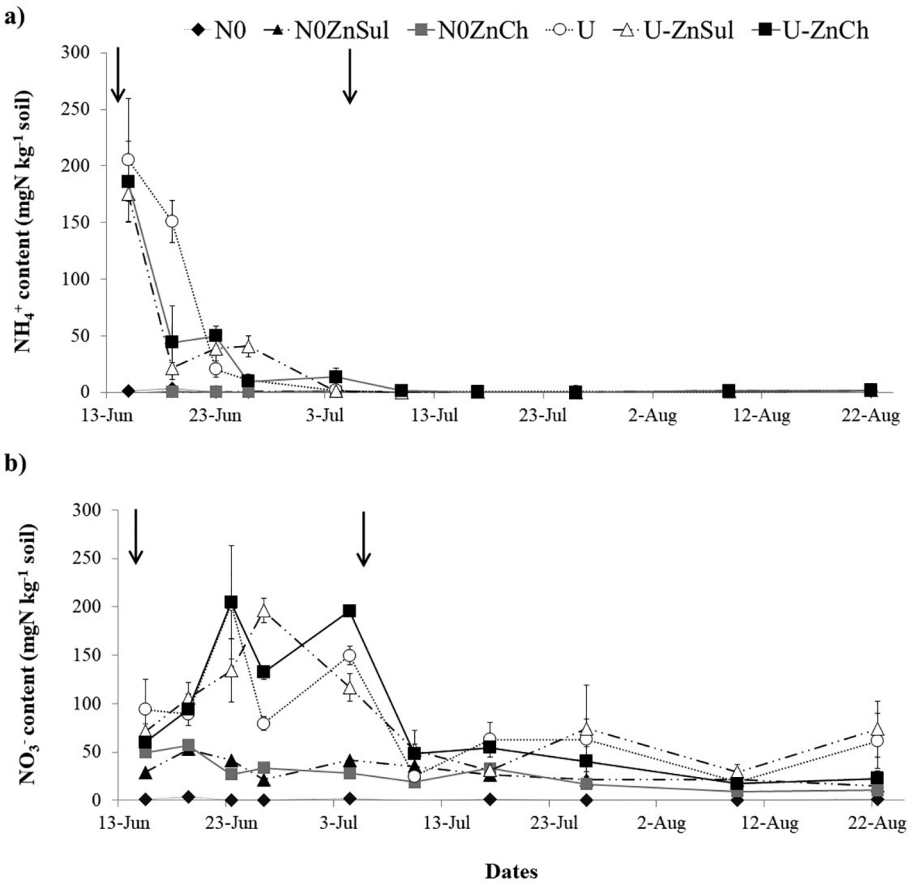

Fig. 2. NH_4^+-N (a) and NO_3^--N (b) content for 68 days after N fertilization for the N application levels of 0 kg N ha^{-1} (N0) and 120 kg N ha^{-1} (U) combined with different Zn sources (control without Zn, Z0, Zn-sulphate, ZnSul, Zn- DTPA-HEDTA-EDTA, ZnCh). The black arrows indicate the Zn-N fertilization (06/14/2017) and the 2nd Zn fertilization event (07/05/2017). Vertical bars indicate standard errors.

Table 2
Total Zn-DTPA and Cu-DTPA concentrations with the different Zn sources (control without Zn, Z0; Zn-sulphate, ZnSul; Zn-DTPA-HEDTA-EDTA, ZnCh) combined with two N application rates (0 kg N ha^{-1}; N0 and 120 kg N ha^{-1}, U) after 1st and 2nd fertilization events and at harvest.

Effect	Zn-DTPA conc.after 1st fertilization (mg kg^{-1})	Zn-DTPA conc.after 2nd fertilization (mg kg^{-1})	Zn-DTPA conc. at harvest (mg kg^{-1})	Cu-DTPA conc.after 1st fertilization (mg kg^{-1})	Cu-DTPA conc. after 2nd fertilization (mg kg^{-1})	Cu-DTPA conc. at harvest (mg kg^{-1})
Nitrogen	*	NS	***	***	***	***
N0	1.55 b	1.27	1.29 b	0.88 b	0.80 b	0.60 b
U	1.34 a	1.66	1.00 a	0.79 a	0.54 a	0.48 a
S.E.	0.05	0.12	0.02	0.01	0.01	0.02
Zinc sources	***	***	***	NS	***	NS
Zn0	0.86 a	0.81 a	0.88 a	0.81	0.62 a	0.53
ZnSul	2.51 b	2.57 c	1.63 b	0.86	0.67 b	0.55
ZnCh	0.96 a	1.03 b	0.92 a	0.85	0.73 c	0.55
S.E.	0.07	0.14	0.03	0.02	0.01	0.02
Zinc × Nitrogen	NS	**	***	*	*	NS
N0Zn0	1.01	0.83 Aa	0.94 Ba	0.89 Ba	0.74 Ba	0.60
U-Zn0	0.71	0.79 Aa	0.82 Aa	0.73 Aa	0.49 Aa	0.45
N0ZnSul	2.66	1.89 Ab	2.00 Bb	0.87 Aa	0.89 Bb	0.60
U-ZnSul	2.35	3.26 Bb	1.25 Ac	0.85 Ab	0.57 Ab	0.50
N0ZnCh	0.97	1.10 Aa	0.92 Aa	0.89 Ba	0.78 Ba	0.61
U-ZnCh	0.95	0.95 Aa	0.92 Ab	0.80 Aab	0.55 Ab	0.49
S.E.	0.09	0.20	0.04	0.03	0.02	0.03

Different letters within columns indicate significant differences by applying the LSD test at $P < 0.05$. Standard Error (S.E.) is given for each effect. *, ** and *** denote significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. "NS" denotes not significant. Different capital letters in the interaction indicate significant differences between N rates within a Zn treatment, whereas different lowercase letters indicate significant differences between Zn treatments within a N rate, by applying the LSD test at $P < 0.05$.

in soil was affected by the N fertilization in the case of ZnSul (after 2nd fertilization and at harvest), but not for ZnCh. The N application resulted in lower Zn availability in soil than did the N0 treatments ($P < 0.05$), both after first fertilization and at harvest.

Concerning the total Cu-DTPA concentrations in soil (Table 2), the Zn-N interaction was statistically significant for the 1st and 2nd fertilization events. The N-fertilized treatments resulted in lower ($P < 0.05$) total Cu-DTPA concentrations than for N0 treatments. In the Zn-treated soils, the Cu-DTPA concentrations were higher in U-ZnSul than in U-Zn0 (1st fertilization) and higher in U-ZnSul and U-ZnCh than in U-Zn0 (2nd fertilization). No significant differences in the total Cu-DTPA content were observed between the N0ZnCh and N0Zn0 treatments after both Zn fertilization events, while concentrations in N0ZnSul were significantly elevated.

3.2. GHG emissions

3.2.1. Nitrous oxide emissions

Nitrous oxide fluxes ranged from −0.28 to 18.35 mg N m^{-2} d^{-1}. The highest peak was reported 7 DAF for treatments with U, particularly for Zn-based treatments (Fig. 3a). A second peak, because of an irrigation event, occurred 12 DAF for U-ZnCh and 16 DAF for U and U-ZnSul treatments. The different fluxes observed for each treatment in the period 0–104 DAF produced significant differences in cumulative N_2O emissions between N-fertilized treatments (Table 3). A significant Zn-N interaction was observed, decreasing cumulative N_2O fluxes in the order U-ZnS > U-ZnCh > U. Differences among Zn treatments were only observed when urea was applied, but not for N0. The N0 treatments resulted in significantly lower cumulative N_2O emissions than did N-fertilized treatments (U), regardless of the type of Zn source.

3.2.2. Methane, soil respiration fluxes and CO_2 equivalent emissions

Negative CH_4 fluxes were generally observed throughout the maize cropping season. No significant differences were observed among N treatments, Zn sources or for the Zn-N interaction regarding cumulative CH_4 oxidation (Table 3). On 21 June (the same day as the N_2O emission peak), the highest CH_4 sink was reached after fertilization (Fig. 3b).

Carbon dioxide emissions (i.e., respiration fluxes from the soil and crop roots) ranged from 0.3 to 3.6 g C m^{-2} d^{-1} (Fig. 3c). The peaks were observed 12 and 5 days after first and second Zn fertilization events, respectively. Regardless of the type of Zn application, the U treatments tended to increase CO_2 fluxes with respect to N0, although the differences were not statistically significant. Zn fertilization resulted in a significant increase in cumulative CO_2 emissions in comparison to the no-Zn application (Table 3).

Concerning the CO_2eq emissions from N_2O and CH_4, fertilization with N exhibited significantly greater net CO_2eq emissions than did the N0 treatments, regardless of method of Zn addition (Table 3). The significant Zn-N interaction effect showed a similar pattern as that for N_2O fluxes. The U-ZnSul treatment produced 50% more CO_2eq emissions than did U, with results for U-ZnCh intermediate.

3.3. Total abundances of nitrifying and denitrifying communities

Total abundances of the bacterial and archaeal 16S rRNA, the bacterial (AOB) and archaeal (AOA) *amoA* gene, and the *nirK*, *norB* and *nosZ* genes (Fig. 4) were only quantified in the N-fertilized treatments because significant differences in N_2O fluxes among Zn sources were only found in U plots (Table 3). During the experimental period, the total abundance of the 16SB was higher than that of the 16SA for all treatments (Fig. 4a, b). After the 1st Zn fertilization event, the Zn-fertilized treatments showed 78% and 77% reductions in 16SB and 16SA abundance, respectively, compared to the U treatment. This effect was maintained for almost three weeks.

During the experimental period, the total abundances of AOB were 79.9% and 90.3% lower for the U-ZnSul and U-ZnCh treatments, respectively, than for the U treatment, and the differences were particularly noteworthy on 4 July (Fig. 4c). Total abundance of AOA was higher for the U treatment than for the U-ZnSul and U-ZnCh treatments only on this second date (Fig. 4d). Differences in abundances were found between bacterial and archaeal *amoA* genes, with the number of copies for AOB statistically higher than that for AOA.

Concerning the denitrification genes, the U treatment resulted in the highest total abundances of *nirK, norB* and, particularly, *nosZ* genes throughout the experiment (Fig. 4e, f and g, respectively). Zinc-fertilized treatments resulted in a reduction in total abundance of denitrifiers, particularly for *nosZ* copies. Accordingly, the ratio between the abundances of *nosZ* and *nirK* genes was significantly higher in the Zn-fertilized than in the U treatment (Fig. 5a). In contrast, the ratio of the abundances of *amoA* to *nosZ* genes was significantly higher in the U

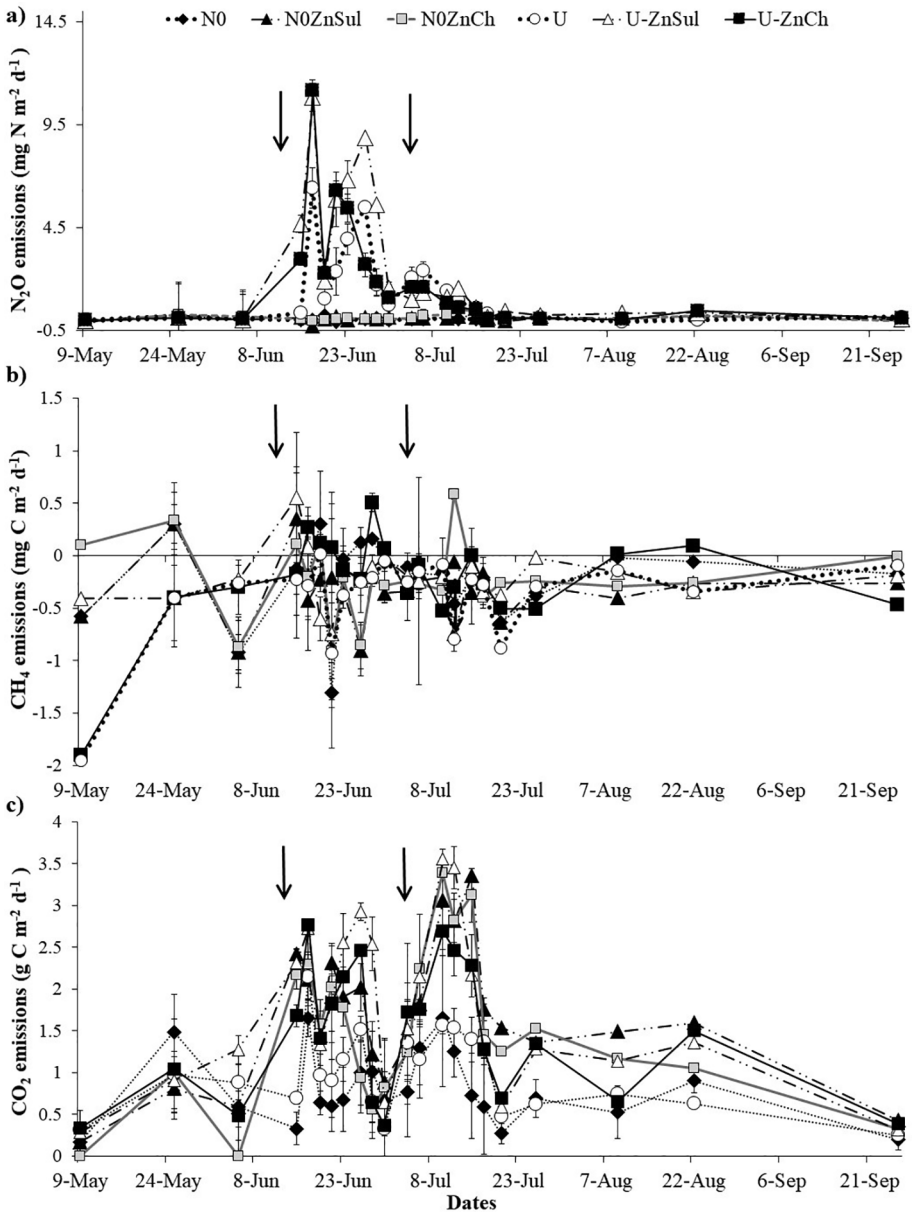

Fig. 3. Daily N_2O (a), CH_4 (b) and soil CO_2 (c) fluxes for N application levels of 0 kg N ha^{-1} (N0) and 120 kg N ha^{-1} (U) combined with different Zn sources (control without Zn, Zn0, Zn-sulphate, ZnSul, Zn- DTPA-HEDTA-EDTA, ZnCh). The black arrows indicate the Zn-N fertilization (06/14/2017) and 2nd Zn fertilization event (07/05/2017). Vertical lines indicate standard errors.

samples than in the U-ZnSul and U-ZnCh treatments (Fig. 5b).

3.4. Agronomic parameters

Total Zn and N concentrations in grain and stover are shown in Table 4. The grain Zn content was significantly elevated with the application of ZnSul fertilizer. However, this was not observed for ZnCh (Table 4). The Zn-N interaction effect was statistically significant for the total Zn concentration in stover; i.e., the U-ZnSul treatment resulted in 28% higher Zn content than in the N0ZnSul treatment, but this was not observed for the other Zn treatments (Zn0 or ZnCh). Fertilization with Zn or N did not cause significant differences in total N concentrations of either grain or stover. The Zn0 and N0 treatments reduced grain yields by 37% and 43% with respect to Zn fertilization and the U treatments, respectively (data not shown). No significant differences in biomass yields were observed among Zn sources, N levels or for the interaction of both factors (data not shown).

The greenhouse gas intensity (GHGI), which was strongly influenced by N_2O emissions, showed that CO_2eq emissions per kilogram of grain yield of the non-N-fertilized treatments was 79% lower on average than for the U treatments (Fig. 6). In addition, the U treatments resulted in lower values of GHGI than for U-ZnCh ($P > 0.05$) and U-ZnSul treatments ($P < 0.05$).

4. Discussion

4.1. Effect of Zn-N co-fertilization on N2O emissions and bacterial gene abundances

The results of this study demonstrate that co-fertilization with urea and a Zn source, either U-ZnSul or U-ZnCh, significantly increased cumulative N_2O emissions in comparison with no Zn application in an irrigated maize crop grown in a calcareous soil under semi-arid conditions (Table 3). This increment of N_2O fluxes was mainly produced during the 30 days following urea application, when the N_2O diffusing to the atmosphere represented more than 80% of the total N_2O emitted

Table 3
Total cumulative N_2O-N, CH_4-C and respiration fluxes and global warming potential (GWP) for the different Zn sources (control without Zn, Zn0; Zn-sulphate, ZnSul; and Zn-DTPA-HEDTA-EDTA, ZnCh) combined with two N application levels (0 kg N ha^{-1}, N0; and 120 kg N ha^{-1}, N120).

Effect	N_2O cumulative emissions (kg N-N_2O ha^{-1})	CH_4 cumulative emissions (kg C-CH_4 ha^{-1})	CO_2 cumulative emissions (kg C-CO_2 ha^{-1})	CO_2eq (N_2O + CH_4) (kg CO_2 ha^{-1})
Nitrogen	***	NS	NS	***
N0	0.15 a	−0.31	1433	33 a
U	1.17 b	−0.43	1446	298 b
S.E.	0.06	0.05	98.68	15.22
Zinc sources	**	NS	***	**
Zn0	0.46 a	−0.42	902 a	114 a
ZnSul	0.86 b	−0.36	1834 b	217 b
ZnCh	0.67 ab	−0.34	1581 b	165 ab
S.E.	0.07	0.06	120.85	18.64
Zinc × Nitrogen	*	NS	NS	**
N0Zn0	0.12 Aa	−0.29	887	26 Aa
U-Zn0	0.80 Ba	−0.55	917	202 Ba
N0ZnSul	0.16 Aa	−0.40	1845	31 Aa
U-ZnSul	1.56 Bc	−0.32	1822	403 Bc
N0ZnCh	0.18 Aa	−0.26	1566	41 Aa
U-ZnCh	1.16 Bb	−0.43	1597	290 Bb
S.E.	0.10	0.09	170.91	26.36

Different letters within columns indicate significant differences by applying the LSD test at $P < 0.05$. Standard Error (S.E.) is given for each effect. *, ** and *** denote significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. "NS" denotes not significant. Different capital letters in the interaction indicate significant differences between N rates within a Zn treatment, whereas different lowercase letters indicate significant differences between Zn treatments within a N rate, by applying the LSD test at $P < 0.05$.

during the experimental period (Fig. 3a). Immediately after any of the 8 irrigation events following urea fertilization, the estimated topsoil WFPS was approximately 80% and decreased over the following 3–4 days to values varying from 20 to 50% (Fig. 1b) due to the high temperatures and evaporation rates (Fig. 1). The related continuous drying-rewetting cycles favoured the occurrence of the nitrification and denitrification pathways, as has been previously reported (Li et al., 2016; Pilegaard, 2013); thus, our results lend support to the hypothesis by Guardia et al. (2017) that coupled nitrification and denitrification are key processes in N_2O production by irrigated soils cultivated with maize in the same region. This is also supported by the rapid decrease in NH_4^+ concentration followed by a decrease in the NO_3^- content (Fig. 2). Focusing on the total amount of N_2O emitted, the N_2O emission factors (EFs) ranged from 0.34% to 0.72%. These values were lower than the 1% default value of the IPCC, but within the range reported by the *meta*-analysis of Cayuela et al. (2017) for irrigated systems (0.63% on average) under Mediterranean conditions. The maximum EFs obtained in the present study were, however, lower than the average values for sprinkler irrigation (0.91%) and maize (0.83%).

Concerning the abundance of nitrifying microbes, which may be highly associated with N_2O emissions under our conditions, the U treatment showed a significant increase in nitrifiers, especially for AOB rather than for AOA. Generally, similar results have been found in N-fertilized soils (e.g., Carey et al., 2016; Ouyang et al., 2018) particularly in alkaline (Jiang et al., 2015) or calcareous pH conditions when organic or synthetic N fertilizers were added (Tao et al., 2017). However, the opposite tendency (i.e., predominance of AOA over AOB) has also been observed in grassland systems (Clark et al., 2020), acidic soils (Jiang et al., 2015) or N-stress (scarcity or overdose) conditions (Duan et al., 2019; Tao et al., 2017). AOB may produce higher N_2O emissions than AOA (Hink et al., 2017). This assumption, coupled with the fact that the abundance of AOB is higher than that of AOA in the conditions of our study, may suggest that the effect of the U treatment on N_2O production from nitrification is mainly due to the bacterial *amoA* gene. Since the high moisture conditions following irrigation (Fig. 1b) also favoured denitrification in the upper part of the soil, the genes involved in this process should also be considered.

Our results showed that the Zn treatments caused a significant decrease (by 77.5% on average) in the total abundances of 16SB and 16SA genes in comparison with the U treatment, at least during the 3 weeks following Zn fertilization. Previous studies have reported a toxic effect of Zn on microbial communities (e.g., Epelde et al., 2008; Kelly et al., 1999). Focusing on nitrifying microorganisms, our results showed that the total abundance of the AOB in the U-ZnSul- and U-ZnCh-treated soils decreased (by 85.1% on average) when compared to the U-amended plots (Fig. 4c). Moreover, the application of ZnCh fertilizer also reduced the total abundance of AOA (Fig. 4d). A negative effect of Zn application on the total abundance of AOA and AOB was also reported by Vasileiadis et al. (2012), who found that AOB are more susceptible than AOA to Zn application. Since similar results have been published for Zn-N co-fertilized soil (Montoya et al., 2018), it is more likely that Zn exerts a larger negative effect on AOB than on AOA. After Zn application, a reduction in ammonia oxidizers was also observed by Kapoor et al. (2015) and Black et al. (2019), who suggested that Zn produces a direct and detrimental effect on nitrifiers which was not observed in control plots. These negative effects of Zn application using synthetic chelates on nitrification rates mediated by the abundance of the *amoA* AOB gene have also been observed by Hu et al. (2003) and Montoya et al. (2018), in the latter study under rainfed semi-arid conditions. In the present investigation, however, significant differences were not found in AOB between the ZnSulf and the ZnCh treatments. Our results also suggest that this effect was not dependent on Zn level, since the lowest mean values of the *amoA* AOB gene ($P > 0.05$) corresponded to the soils treated with 0.30 kg Zn ha^{-1} ZnCh rather than the treatment with 10 kg Zn ha^{-1} ZnSul.

Zn-N co-fertilization also resulted in a reduction in the total abundance of the *nosZ* gene and, to a lesser extent, in that of *nirK*. These results did not agree with those of Montoya et al. (2018), who reported that fertilization with U-ZnCh of a wheat crop growing under rainfed semi-arid conditions increased the total abundance of *nosZ* with respect to the U treatment, thus resulting in a 21.4% abatement of cumulative N_2O emissions (which was also attributed to a significant effect on ammonia oxidizers). The lopsided decrease in the abundance of both genes (*nirK* involved in N_2O production and *nosZ* involved in N_2O consumption) may have been critical for explaining the higher cumulative N_2O emissions in Zn-N fertilized plots. In the present study, the *nirK/nosZ* ratio was significantly lower in the U treatment (0.21 on average) than in the U-ZnCh (1.00 on average) and U-ZnSul (0.92 on average) treatments, thus suggesting that complete denitrification (which could be relevant due to the high WFPS reached, Fig. 1b) was less favoured under Zn-N co-fertilization than with N fertilization alone. These findings agree with those of Ruyters et al. (2010), who found that Zn application exerted a stress on the *nosZ*-harbouring communities, thus leading to an inhibitory effect on N_2O reduction. We hypothesize that the environmental conditions (i.e., high soil temperatures and drying-rewetting cycles resulting in high WFPS values) caused rapid nitrification of NH_4^+ (Fig. 2a), making the denitrification process during N_2O peaking more relevant (Fig. 3a) than under rainfed conditions such as those in Montoya et al. (2018). Under conditions in the present study, the imbalance between N_2O producers (i.e., denitrifiers containing the *nirK* and/or *norB* genes) and N_2O consumers (i.e., those harbouring the *nosZ* gene) could have been a key driver for N_2O losses after Zn fertilization. These continuous and cycling fluctuations in soil moisture and redox potential may have a critical influence on N_2O emissions from denitrification and coupled nitrification–denitrification when trace metals are added, so further research is needed to explore these relationships at a biochemical scale.

Copper is a cofactor for the AmoA, NirK and NosZ enzymes (Glass and Orphan, 2012) and its availability in soils has been shown to affect

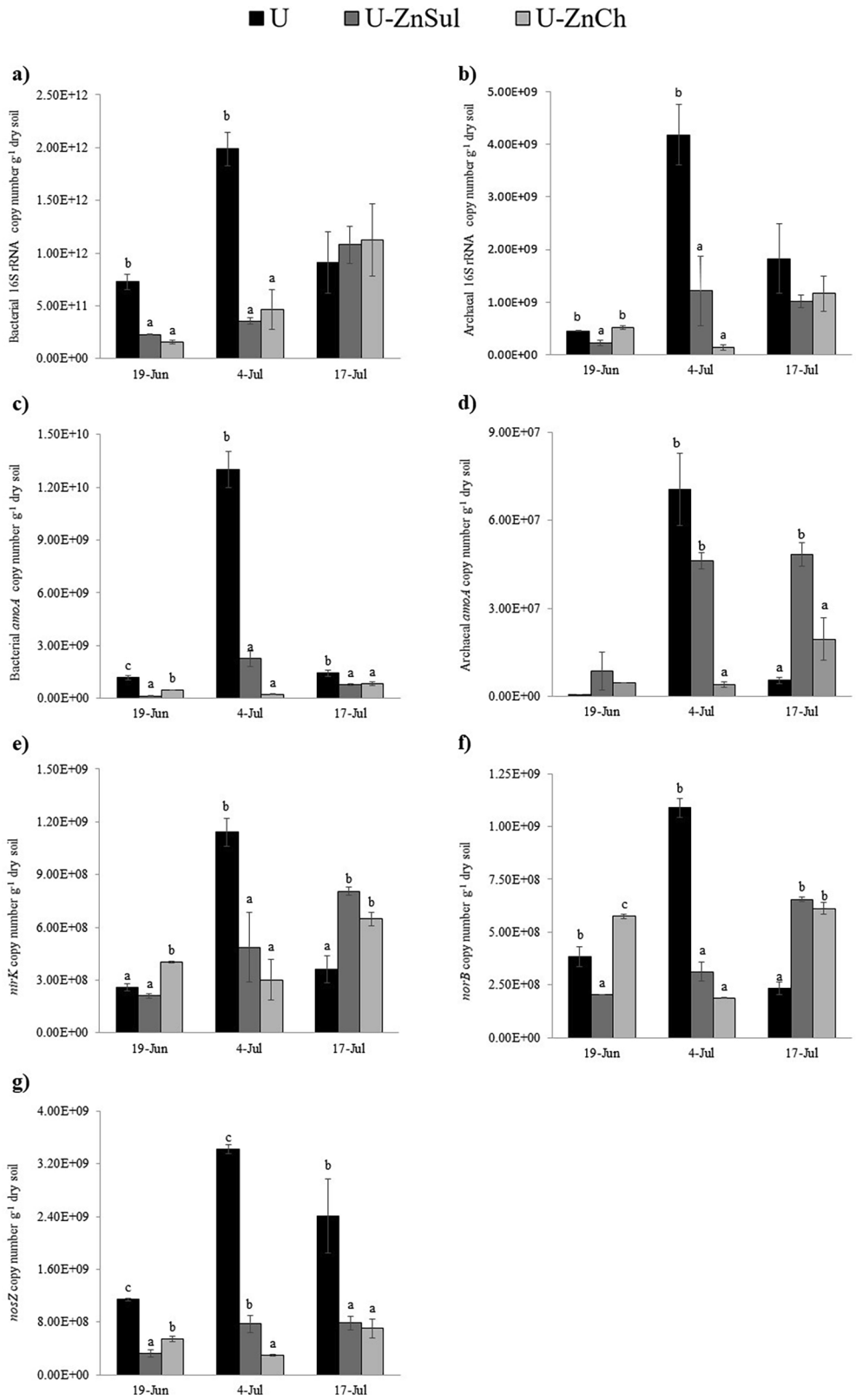

Fig. 4. Numbers of copies of 16SB (a), 16SA (b), the *amoA* gene from AOB (c) and from AOA (d), and *nirK* (e), *norB* (f) and *nosZ* (g) genes in three sampling periods for the N rate of 120 kg N ha^{-1} (U) combined with different Zn sources (control without Zn, Zn0; Zn-Sulphate, ZnSul; Zn-DTPA-HEDTA-EDTA, ZnCh). Statistical differences at $P < 0.05$ (LSD test) are indicated by different letters. Vertical lines indicate the standard deviation from the mean. The scale of the Y-axes has been adapted in each case to improve the visualization of the data.

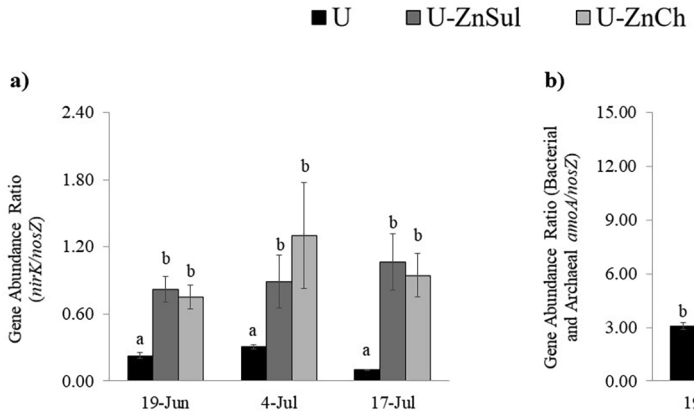

Fig. 5. Abundance ratios of genes functioning in denitrification and nitrification and nitrous oxide reduction. (a) *nirK/nosZ* ratio and (b) AOB + AOA/*nosZ* ratio, in three sampling periods for the N level of 120 kg N ha^{-1} (U) combined with different Zn sources (control without Zn, Zn0; Zn-Sulphate, ZnSul; Zn-DTPA-HEDTA-EDTA, ZnCh). Statistical differences at $P < 0.05$ (LSD test) are indicated by different letters. Vertical lines indicate the standard deviation from the mean. The scale of the Y-axes has been adapted in each case to improve the visualization of the data.

Table 4
Total Zn and N concentrations in grain and stover for different Zn sources (control without Zn, Zn0; Zn-sulphate, ZnSul; Zn-DTPA-HEDTA-EDTA, ZnCh) at two N application levels (0 kg N ha^{-1}, N0; and 120 kg N ha^{-1}, U).

Effect	Total Zn conc. Grain (mg kg^{-1})	Total Zn conc. Stover (mg kg^{-1})	Total N conc. Grain %	Total N conc. Stover %
Nitrogen	NS	NS	NS	NS
N0	15.14	33.40	1.76	1.10
U	15.45	39.76	1.71	1.02
S.E.	0.34	2.46	0.02	0.06
Zinc sources	*	***	NS	NS
Zn0	14.48 a	10.73 a	1.75	1.10
ZnSul	16.45 b	81.39 b	1.71	1.04
ZnCh	14.96 a	17.62 a	1.74	1.05
S.E.	0.41	3.02	0.03	0.08
Zinc × Nitrogen	NS	*	NS	NS
N0Zn0	14.34	12.32 Aa	1.81	1.15
U-Zn0	14.61	9.14 Aa	1.69	1.05
N0ZnSul	16.17	68.16 Ab	1.69	1.05
U-ZnSul	16.72	94.61 Bb	1.70	1.04
N0ZnCh	14.89	19.72 Aa	1.78	1.11
U-ZnCh	15.03	15.53 Aa	1.69	0.98
S.E.	0.59	4.27	0.03	0.11

Different letters within columns indicate significant differences by applying the LSD test at $P < 0.05$. Standard Error (S.E.) is given for each effect. *, ** and *** denote significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. "NS" denotes not significant. Different capital letters in the interaction indicate significant differences between N rates within a Zn treatment, whereas different lowercase letters indicate significant differences between Zn treatments within a N rate, by applying the LSD test at $P < 0.05$.

the abundance of the *nosZ* gene (Sullivan et al., 2013). However, Zn-N plots resulted in higher available Cu in the soil than did the U treatment, thus failing to explain the reduction of *amoA*, *nirK* and *nosZ* genes. The temporary changes in redox potential could have affected the predominant Cu form and therefore its availability as a co-factor, so it will be necessary to explore in future experiments whether DTPA-extractable Cu (the same applies for Zn and Fe) is the most adequate indicator.

4.2. Effect of Zn-N co-fertilization on CH_4 and CO2 emissions

As reported for many non-flooded agricultural soils (Aronson and Helliker, 2010), net CH_4 sink status was determined for most of the soil gas samples analysed in this study (Fig. 3b). No differences in cumulative CH_4 emissions were found after N or Zn application. Aerobic methanotrophs are largely dependent not only on Cu-containing metalloenzymes but also on other metals such as Fe and Zn (Glass and Orphan, 2012). In this study, the small and temporary differences in Cu and Zn availability in soil between N levels or Zn sources (see Section 3.1) did not influence the cumulative CH_4 oxidation rates (Table 3), as observed by Montoya et al. (2018) for winter wheat.

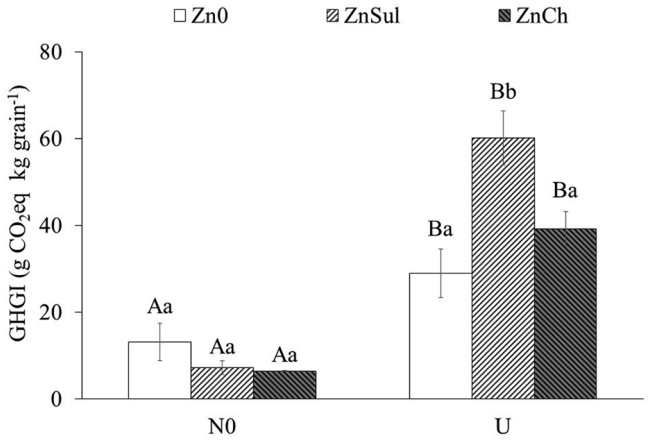

Fig. 6. Greenhouse gas intensity (GHGI) for the different Zn sources (control without Zn, Zn0; Zn-sulphate, ZnSul; and Zn-DTPA-HEDTA-EDTA, ZnCh) at two N application levels (0 kg N ha^{-1}, N0; and 120 kg N ha^{-1}, N120). Statistical differences in the Zn-N interaction effect at $P \leq 0.05$ (LSD test) are indicated by different capital letters for significant differences between N levels within a Zn treatment, whereas different lowercase letters signify significant differences between Zn treatments within the same N level. The vertical line on each bar represents the standard deviation from the mean.

Despite Zn fertilization reducing total abundances of the bacterial and archaeal populations, an increase in CO_2 emissions was observed (Fig. 4 and Table 3). Because root respiration contributes 92% to the total soil respiration during the vegetative stage of maize (V6-V8) (Hu et al., 2008), it is possible that the negative effect of Zn on microbial abundance was masked by root respiration, which could have been enhanced by Zn application. Indeed, Zhang et al. (2020) reported that Zn fertilization significantly increased root growth, thus improving water and nutrient uptake under deficit irrigation conditions. With regards to the temporal evolution of respiration fluxes, the effect of soil rewetting on CO_2 pulses (Barnard et al., 2020; Liang et al., 2016) was observed at the beginning of July (Fig. 3c), after a sharp decline of daily emissions due to high soil temperatures and low soil moisture (Figs. 1, 2), which limited the activity of soil microbiota.

The CO_2eq emissions and GHGI exhibited patterns similar to those found for N_2O emissions after Zn fertilization, being more closely related to the pattern obtained after the highest application of the ZnSul fertilizer. These results contrast with those of Montoya et al. (2018), who reported that Zn chelate-based fertilizers reduce the total CO_2eq emissions of a rainfed wheat crop. According to our results, the use of Zn fertilization should not be recommended to minimize the ratio of N_2O emissions per kilogram of grain yield in irrigated maize cropping

systems in Mediterranean areas.

4.3. Response of Zn-N co-fertilization in irrigated maize

Increasing the Zn concentration in maize grain through biofortification is important for humans and livestock, and a concentration higher than 22.1 mg kg^{-1} has been suggested by the USDA Nutrient Database (http://ndb.nal.usda.gov/ndb/) to produce biofortification in maize. The mean Zn content in grains from plants not treated with Zn was 14.5 mg kg^{-1}, and only the ZnSul treatment caused a significant 12% increase in Zn content (Table 2). In a previous study, treatment of maize plants with soil-foliar ZnSul also increased the Zn content of the grains by 9% (Cakmak and Kutman, 2018). Effective foliar application of fertilizers to maize during the late growth stage, which is the most effective for Zn biofortification (Liu et al., 2017), is difficult due to the special growth characteristics of the plants. This is why, regardless of the Zn source, availability of Zn in the soil at this growth stage is required to increase the Zn concentration in the grains. In this study, the application of Zn fertilizers (independently of Zn source) made it possible to reach the Zn availability threshold, although a 2nd foliar-soil fertilization was necessary for the ZnCh fertilizer to surpass the critical value of soil Zn deficiency ($\leq$1 mg Zn kg^{-1} soil) (Table 2).

During senescence, maize is known to translocate Zn to grain, N being the main factor stimulating this process according to Barunawati et al. (2013). In this sense, our results showed that the N content in grain after Zn-N application was not significantly different than that of unfertilized plants. The lack of significant differences in grain N concentrations between N0 and U can be explained by the broadly reported trade-off between grain yield and N concentration in grain (Savin et al., 2019). In fact, an important fraction of the Zn was maintained in stover (Table 4). The combination of U and the ZnSul fertilizer (U-ZnSul treatment) produced a 38.91% increment over the value found for the N0ZnSul treatment. Considering that maize stover is often used as ruminant feed, a high Zn concentration is of interest for the formulation of animal diets. The application of stover as a C source to soils with low Zn availability could also help to improve the availability of Zn in soil for the subsequent crop in the rotation. Soil organic matter may play an important role in mediating Zn availability (Alloway, 2008), because these organic fractions can release soluble Zn complexes and thus favour Zn availability (Alvarez and Gonzalez, 2006), particularly under arid or semi-arid calcareous soils with low organic C contents (Moreno-Jiménez et al., 2019). Therefore, the management of crop residues aiming to increase the organic matter content in semi-arid soils should be encouraged to improve the availability of Zn and other micronutrients while enhancing soil quality and promoting net C sequestration.

5. Conclusions

Our results suggest that the effect of Zn fertilization on N_2O emissions may be highly dependent on the relative abundance of *nosZ* (N_2O reducing) genes in comparison to other denitrification genes (N_2O producing), when environmental conditions are favourable for coupled nitrification–denitrification (drying-rewetting episodes), and high WFPS values conducive to complete denitrification are temporarily reached. Elevated N_2O emissions were observed for both Zn sources (particularly in the $ZnSO_4$-amended subplots which received a higher Zn rate), and a similar tendency was observed for respiration fluxes (in spite of the lower gene abundances), possibly as a result of the enhancement of root biomass. Our findings in this experiment did not confirm our initial hypothesis based on the results under rainfed conditions (i.e., Montoya et al., 2018), in which the effect of Zn fertilization was source-dependent, and an increase in *nosZ* abundances was observed for the synthetic chelate. Further research under different irrigation systems and management (rate, frequency) is therefore needed, exploring in depth the temporary changes in the availability of metal co-factors and active nitrifying and denitrifying communities. In addition, the accuracy in the detection of GHG emission pulses could be improved using high temporal resolution techniques that allow continuous measurements.

Our results show that even though utilization of micronutrient fertilizers alleviated soil Zn deficiency, these products conflicted with the pivotal goal of reducing N_2O losses without positive side-effects regarding Zn biofortification or crop yield enhancement under the conditions of our study (i.e., irrigated maize in semi-arid climate). The use of ZnSul rather than ZnCh is recommended due to its potential to achieve Zn biofortification in grain (even though the target value of 22.1 mg Zn kg^{-1}, previously suggested as a threshold for grain Zn biofortification, was not reached). Moreover, additional agricultural practices regarding N and water management would be necessary to prevent an increase in N_2O emissions from $ZnSO_4$ application under the conditions of our study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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